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Dynamic Changes in Dopaminergic Neurotransmission Induced by a Low Concentration of Bisphenol-A in Neurones and Astrocytes

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Key words: bisphenol-A, neurone, astrocyte, dopamine, 17β-oestradiol, drug abuse.

Abstract

One of the most common chemicals that behaves as an endocrine disruptor is the compound 4,4′-isopronylidenediphenol, called bisphenol-A (BPA). We previously reported that prenatal and postnatal exposure to BPA potentiated central dopaminergic neurotransmission, resulting in supersensitivity to psychostimulant-induced pharmacological actions. Many recent findings have supported the idea that astrocytes, which are a subpopulation of glial cells, play a critical role in neuronal transmission in the central nervous system. The present study aimed to investigate the role of neurone–astrocyte communication in the enhancement of dopaminergic neurotransmission induced by BPA. We found that treatment of mouse purified astrocytes and neurone/glia cocultures with BPA *in vitro* caused the activation of astrocytes, as detected by a stellate morphology and an increase in levels of glial fibrillary acidic protein. A low concentration of BPA significantly enhanced the Ca^{2+} responses to dopamine in both neurones and astrocytes. Furthermore, a high concentration of BPA markedly induced the activation of caspase-3, which is a marker of neuronal apoptotic cell death in mouse midbrain neurone/glia cocultures. By contrast, treatment with 17β-oestradiol (E_2) had no such effects. Prenatal and neonatal exposure to BPA led to an enhancement of the dopamine-dependent rewarding effect induced by morphine. These findings provide evidence that BPA alters dopamine responsiveness in neurones and astrocytes and that, at least in part, it may contribute to potentiate the development of psychological dependence on drugs of abuse.

The foetus uses natural hormonal messages that originate in its own hormone system and that of its mother for developmental guidance. Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. One of the most common endocrine disruptors is the compound 4,4′-isopronylidenediphenol, called bisphenol-A (BPA), which is used in the manufacture of many types of products. These include polycarbonate plastic food storage containers (i.e. baby bottles and water carboys), the lining of food or beverage cans (1, 2), dental composites and sealants and a bioactive bone cement, indicating the potential for human exposure to BPA in daily life.

Our recent studies suggest that exposure to BPA during prenantal and postnatal development has long-lasting effects on central dopaminergic systems linked with behavioural rewarding effects, as well as drug addiction and the reward induced by drugs of abuse (3, 4). The dopamine projection most often linked with a behavioural-rewarding effect is the mesolimbic dopamine system, which originates from the ventral tegmental area (VTA) and terminates at the nucleus accumbens.

We previously demonstrated that prenatal and neonatal exposure to BPA markedly enhanced the rewarding effects induced by drugs of abuse, such as methamphetamine (5) and morphine (6). We also demonstrated that, in adult mice, prenatal and neonatal exposure to BPA enhanced function mediated by central D_1 receptors, which play a substantial role in the rewarding effect of drugs of abuse (5). These treatments also attenuated dopamine function mediated by the D_3 receptor subtype that contributes to the inhibitory modulation of D_1/D_2 receptor-mediated signalling (7, 8). These findings indicate that exposure to BPA during development alters dopaminergic neurotransmission in the central nervous system (CNS), which results in enhancement of the psychological dependence on drugs of abuse. However, the mechanisms underlying these enduring effects of BPA are unknown.

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Many toxic stimuli activate astrocytes, as determined by morphological changes and by an increase in the levels of glial fibrillary acidic protein (GFAP), which is a marker of astrocytes (9, 10). The activation of astrocytes may control the structural and functional plasticity of synapses in the CNS. However, long-term exposure to drugs of abuse can induce neuronal plasticity (11, 12), and we have shown that treatment of mouse cortical neurone/glia cocultures with drugs of abuse, such as methamphetamine and morphine, caused morphological changes in astrocytes (13). Moreover, treatment with methamphetamine increased the sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids (13). Together, these findings indicate that astrocytes may play an important role in the development of dependence on drugs of abuse.

Although BPA may affect dopaminergic signalling in the CNS, little is known about the role of BPA in neurone-astrocyte communication. The present study aimed to clarify the effect of BPA in neurone-glia communication. We used mouse midbrain neurone/glia cocultures and purified astrocytes to determine the effects of BPA in the mesolimbic dopamine system.

Because the sex steroid hormones (oestrogens and androgens) have been shown to exert profound effects on brain differentiation, neural plasticity and central neurotransmission (14, 15) and BPA has an affinity for oestrogen receptors, albeit 1 : 2000 that of 17β-oestradiol (E_2) (1), we also investigated the effect of E_2 on astrocytic and neuronal responses and the rewarding effect induced by morphine.

Materials and methods

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Tissue processing

Purified midbrain astrocytes

Midbrains were dissected from ICR mice at postnatal 1 day (Tokyo Laboratory Animals Science, Tokyo, Japan), minced, and treated with trypsin (0.025%; Invitrogen, Grand Island, NY, USA) dissolved in phosphate-buffered saline (PBS) solution containing 0.02% L-cysteine monohydrate (Sigma-Aldrich, St Louis, MO, USA), 0.5% glucose (Wako Pure Chemicals, Osaka, Japan) and 0.02% bovine serum albumin (Wako Pure Chemicals). After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (20 min, 1000 *g*). After centrifugation, cells were plated in a flask (75 cm^2 culture flask; Corning Inc., Corning, NY, USA). Seven days after seeding in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% precolostrum newborn calf serum (FBS, Invitrogen), 5% heat-inactivated (56 °C, 30 min) horse serum (HS, Invitrogen), 10 U/ml penicillin and 10 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C, the flask was shaken for 12 h at 37 °C to remove nonastrocytic cells. Seven days after seeding, the cells were seeded at a density of 1×10^5 cells/cm^3, and maintained for 7 days in DMEM supplemented with 5% FBS, 5% HS, 10 U/ml penicillin and 10^{-5} g/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Midbrain neurone/glia cocultures

Midbrains were obtained from newborn ICR mice at postnatal 1 day, minced, and treated with papain (9 U/ml; Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% bovine serum albumin. After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (10 min, 1000 *g*). The cells were then seeded at a density of 2×10^6 cells/cm^3. The cells were maintained for 7 days in DMEM supplemented with 10% FBS, 10 U/ml penicillin and 10 μg/ml streptomycin. Eight days after seeding, the cells were treated with drugs. In this culture condition, NeuN-positive neurones are surrounded by astrocytes (16).

Drug treatment and immunohistochemistry

Eight days after seeding, *in vitro*, the cells were treated with either normal medium or medium containing bisphenol A (BPA; 10 fM to 1 μM; Wako Pure Chemicals) or 1,3,5[10]-estratriene-3,17β-diol (E_2, 10 fM to 1 μM; Sigma-Aldrich) for 24 h. To explore role for steroid hormone receptors in mediating the effects of BPA (1 pM or 1 μM, 24 h), cells were pretreated with either an oestrogen receptor antagonist 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]-nonyl]estra-1,3,5(10)-triene-3.17-diol (ICI182,780; 100 nM, 1 μM or 2 μM; Tocris-Cookson, Ellisville, MO, USA), an oestrogen receptor agonist/antagonist tamoxifen (100 nM, 1 μM or 10 μM; Sigma-Aldrich), a progesterone receptor antagonist mifepristone (100 nM, 1 μM or 10 μM; Sigma-Aldrich) or an androgen receptor antagonist flutamide (100 nM, 1 μM or 10 μM; Sigma-Aldrich) for 24 h. Cells were then treated with normal medium or BPA (1 pM, 1 mM) with or without these steroid hormone receptor ligands for an additional 24 h. Glial cells were then identified by immunofluorescence using mouse anti-glial fibrillary acidic protein antibody (GFAP, dilution 1 : 1000; Chemicon Inc., Temecula, CA, U.S.A), rabbit anti-GFAP (dilution 1 : 1000, Chemicon) or mouse anti-neuronal nuclei (Neu-N) antibody (dilution 1 : 1000, Chemicon) followed by incubation with Alexa 488-conjugated goat anti-mouse IgG (dilution 1 : 4000), Alexa 488-conjugated goat anti-rabbit IgG (dilution 1 : 4000) or Alexa 546-conjugated goat anti-mouse IgG (dilution 1 : 4000). Images were collected using a Radiance 2000 laser-scanning microscope (Radiance 2000; Bio-Rad, Carlsbad, CA, USA).

The intensity of GFAP-like immunoreacivity was measured with a computer-assisted system (NIH Image, Bethesda MD, USA). The upper and lower threshold intensity ranges were adjusted to encompass and match the immunoreactivity to provide an image with immunoreactive material appearing in black pixels, and nonimmunoreactive material as white pixels. The area and intensity of pixels within the threshold value representing immunoreactivity were calculated. We randomly chose 10 areas (80 × 80 pixels) for calculation of GFAP-like immunoreactivity in each image (512 × 512 pixels). The experiments were repeatedly performed by at least three independent culture preparations. The intensity of GFAP-like immunoreactivity was expressed as a percent increase (mean ± SEM) with respect to that in control cells, which were seeded on the same plate.

To evaluate the apoptotic neuronal cell death, mouse midbrain neurone/glia cocultures were treated with normal medium, BPA (1 pM, 1 nM or 1 μM) or E_2 (1 pM, 1 nm or 1 μM) for 24 h. The cells were then identified by immunofluorescence, using rabbit-anticleaved caspase-3 antibody (dilution 1 : 100; Cell Signaling Technology Inc., Beverly, MA, USA), followed by incubation with Alexa 488 conjugated goat anti-rabbit IgG (dilution 1 : 10000). Images were collected using a Radiance 2000 laser-scanning microscope.

Confocal Ca^{2+} imaging

Confocal Ca^{2+} imaging was conducted as previously described (13, 16). Mouse midbrain neurone/glia cocultures or purified astrocytes were incubated for 24 h with normal medium or medium containing BPA (1 pM, 1 nM or 1 μM). Cells were then loaded with 10 μM fluo-3 acetoxymethyl ester (Dojindo Molecular Technologies, Kumamoto, Japan) during a 90-min incubation at room temperature. After a further 20–30 min of de-esterification with the acetoxymethyl ester, the cells which seeded on coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance 2000). Fluo-3 was excited with the 488-nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths > 515 nm, and average baseline fluorescence (F_0) of each cell was calculated. To compensate for the uneven distribution of fluo-3, self-ratios were calculated (ratio: $R_s = F/F_0$). The amplitude was determined by subtracting the average of baseline fluorescence ratio (F_{basal}/F_0) from the maximum of fluorescence ratio after a drug treatment (F_{max}/F_0). Dopamine (1, 10 or 100 μM; Sigma-Aldrich) was perfused via a plastic tube for 30 s at 5 ml/min at room temperature in cultured cortical neurones or astrocytes followed by superfusion of basal salt solution (BSS,

pH 7.4) containing 150 mM of NaCl, 5 mM of KCL, 1.8 mM of $CaCl_2$, 1.2 mM of $MgCl_2$, 25 mM of N-2-hydroxyethylpiperazine-N'-2-ethaneslufonic acid and 10 mM of D-glucose.

Prenatal and neonatal exposure to BPA

All experiments were performed using male ddY mice (7 weeks old) (Tokyo Laboratory Animals Science) that had been indirectly exposed prenatally and neonatally to BPA, administered to their mothers.

Mice were orally administered either olive oil (control; 0.1 mg/kg), BPA (3 μg/kg/day or 200 mg/kg/day) dissolved in olive oil (Wako Pure Chemicals) or E_2 (3 μg/kg/day) through the stomach sonde. Female mice (10 weeks old) were orally treated with these chemicals three times a day (08.00, 14.00 and 20.00 h): from mating to weaning. Therefore, these chemicals were administered during pregnancy (20 days) and lactation (21 days, total 41 days).

Place conditioning

The place-conditioning procedure has been used to evaluate the motivation properties, such as rewarding or aversive effects, of drugs in adult rodents (17). Place conditioning was conducted as previously described (5, 6). The apparatus was a shuttle box (15 × 30 × 15 cm: w × l × h), which was made of acrylic resin board and divided into two equal-sized compartments. One compartment is white with a textured floor, and the other is black with a smooth floor to create equally preferred compartments. For conditioning, groups of mice (seven mice in a group) were confined to one compartment after morphine injections (morphine-paired side) and to the other compartment after saline injection (saline-paired side). The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. Conditioning sessions (3 days for morphine, 3 days for saline) were conducted once daily for 6 days. Immediately after s.c. injection of morphine (1 mg/kg), animals were placed in one compartment for 1 h. On alternate days, animals receiving the vehicle were placed in the other compartment for 1 h. On day 7, tests of conditioning were performed. The partition separating the two compartments was raised to 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. The mice were not treated with either morphine or saline, and then placed on the platform. The time spent in each compartment during a 900-s session was then recorded automatically using an infrared beam sensor (KN-80, Natsume Seisakusyo Co., Tokyo, Japan). All sessions were conducted under conditions of dim illumination (28 lux lamp) and white masking noise.

Statistical analysis

Data for GFAP-like immunoreactivity and confocal Ca^{2+} imaging are presented as the mean ± SEM. The statistical significance of differences between the groups were assessed by one-way analysis of variance (ANOVA) followed by Student's t-test.

Conditioning scores for each mouse were obtained by subtracting the cumulative time spent in the saline-paired side from that in the morphine-paired side, and are expressed as means ± SEM. Statistical analysis for the place conditioning study was conducted using one-way ANOVA followed by Bonferroni/Dunnett's test.

Results

BPA, but not E_2, causes the activation of astrocytes

To ascertain the effect of BPA in mouse purified midbrain astrocytes, we performed immunohistochemical staining with a polyclonal antibody for GFAP. The results showed a biphasic response. Mouse midbrain purified astrocytes were treated with either normal medium or BPA (BPA: 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM) for 24 h. Treatment with BPA (100 fM, 1 pM, 10 pM, 10 nM, 100 nM or 1 μM) for 24 h caused a robust activation of mouse purified midbrain astrocytes, as detected by a stellate morphology and an increase in the levels of GFAP-like immunoreactivity ($P < 0.001$ versus control cells) (Fig. 1A,B). On the other hand, treatment with the mid-range dosed of BPA (10 fM, 100 pM, 1 nM) for 24 h failed to produce morphological changes in mouse purified midbrain astrocytes (Fig. 1A,B).

Unlike BPA, treatment with E_2 (10 fM to 1 μM, 24 h) failed to produce morphological changes in midbrain astrocytes at all concentrations tested (Fig. 1C,D).

We next explored the effect of BPA on mouse midbrain neurone/glia cocultures. In this culture system, numerous glial cells, especially astrocytes, surround neurones. Mouse midbrain neurone/glia cocultures were treated with either normal medium or BPA (BPA: 10 fM to 1 μM) for 24 h. In neurone/glia cocultures, BPA caused biphasic activations of astrocytes. Treatment with BPA (100 fM, 1 pM, 10 pM, 100 nM or 1 μM, 24 h) caused a robust activation of astrocytes in midbrain neurone/glia cocultures (*$P < 0.05$, **$P < 0.01$, *** $P < 0.001$ versus control cells) (Fig. 2A,B), whereas treatment with BPA (10 fM, 100 pM, 1 nM or 10 nM, 24 h) failed to produce an increase in GFAP-like immunoreactivity in mouse midbrain neurone/glia cocultures. E_2 (10 fM to 1 μM) failed to produce an increase in GFAP-like immunoreactivity in mouse midbrain neurone/glia cocultures at any doses tested (Fig. 2C,D).

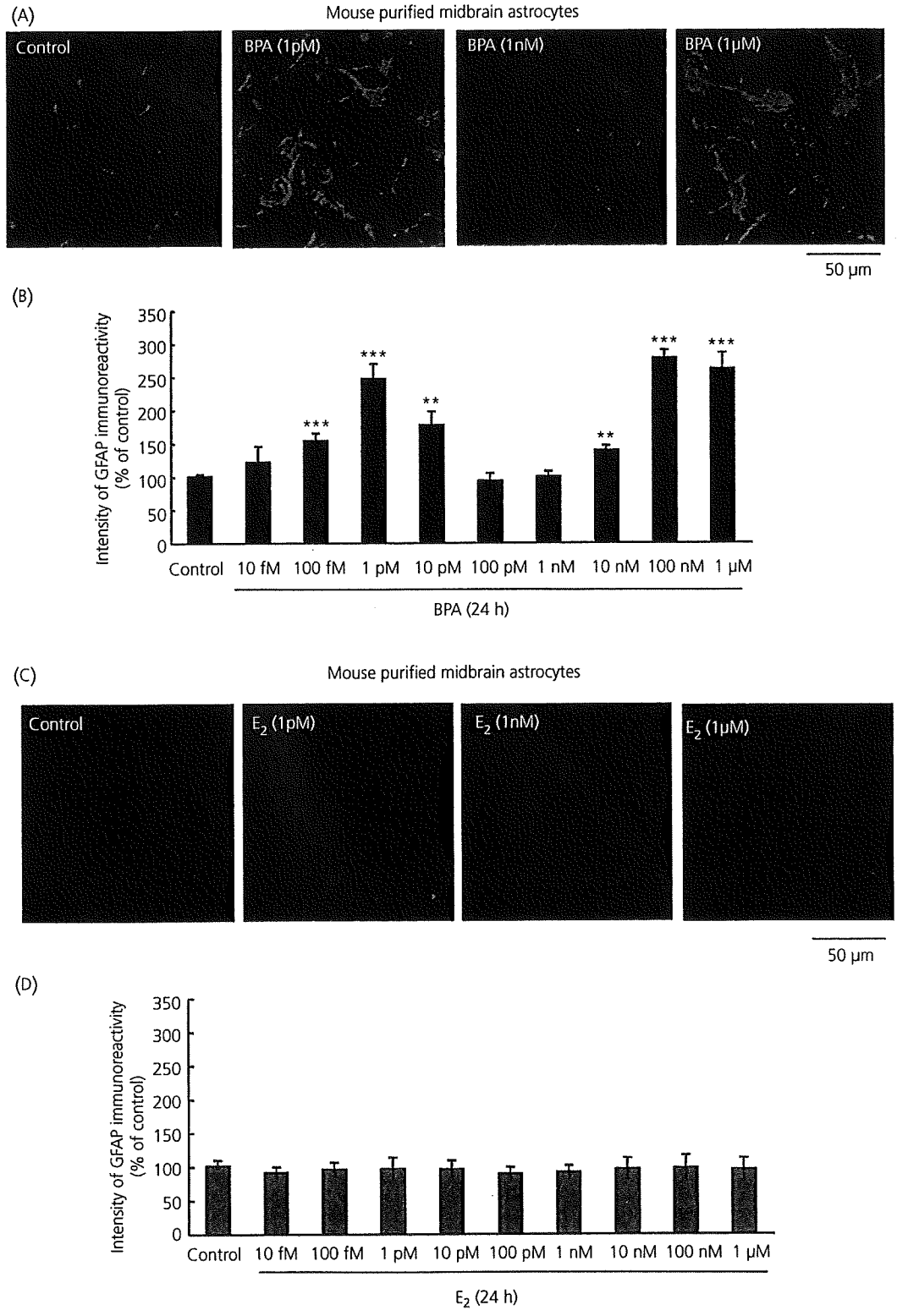
Enhancement of dopamine-induced Ca^{2+} responses by BPA

Dopamine (1–100 μM) produced a transient increase in the intracellular Ca^{2+} concentration in mouse purified midbrain astrocytes (Fig. 3). The Ca^{2+} responses to dopamine (100 μM) in astrocytes were significantly enhanced by pretreatment with a low concentration of BPA (1 pM, 24 h, **$P < 0.01$ versus control cells) (Fig. 3). By contrast, treatment with a high concentration of BPA (1 nM or 1 μM, 24 h) had no effect on the Ca^{2+} responses to dopamine in mouse purified midbrain astrocytes (Fig. 3).

Using immunocytochemical methods shown in Fig. 4(A) according to the study described previously (16), Neu-N-positive neurones are surrounded by GFAP-positive astrocytes in mixed neurone/glia cocultures. On the basis of morphological appearance, neurone-like cells were selected for the Ca^{2+} imaging studies. Under these criteria, dopamine (1–100 μM) produced a transient increase in the intracellular

FIG. 1. Treatment with bisphenol-A (BPA) for 24 h caused biphasic astrocytic activation in mouse purified midbrain astrocytes. (A) Mouse purified midbrain astrocytes were treated with normal medium or BPA (1 pM to 1 μM). The cells were stained with a polyclonal antibody to glial fibrillary acidic protein (GFAP). (B) Mouse purified midbrain astrocytes were treated with normal medium or BPA (10 fM to 1 μM) for 24 h and stained with a polyclonal antibody to GFAP. The intensity of GFAP-immunoreactivity from ten areas in each image was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells. The experiments were repeatedly performed by at least three independent culture preparations. **$P < 0.001$, ***$P < 0.001$ versus control cells. (C) Mouse purified midbrain astrocytes were treated with normal medium or 17β-oestradiol (E_2, 1 pM to 1 μM). The cells were stained with a polyclonal antibody to GFAP. (D) Mouse purified midbrain astrocytes were treated with normal medium or E_2 (10 fM to 1 μM) for 24 h and stained with a polyclonal antibody to GFAP. The intensity of GFAP-immunoreactivity from ten areas in each image was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells. The experiments were repeatedly performed by at least three independent culture preparations.

Ca^{2+} concentration in cultured midbrain neurone-like cells (Fig. 4B,C). These Ca^{2+} responses were significantly enhanced by treatment with a low concentration of BPA (1 pM, 24 h, *P < 0.05, **P < 0.01, ***P < 0.001 versus control cells) (Fig. 4B,C). Treatment with a high concentration of BPA (1 nM, 24 h) had no effect on the Ca^{2+} response to any



concentrations of dopamine, whereas the highest concentration of BPA (1 μM) suppressed the Ca^{2+} response to 100 μM dopamine (***P < 0.001 versus control cells) (Fig. 4B,C).

Effects of steroid hormone antagonists on the activation of astrocytes induced by BPA

To explore the involvement of steroid hormone receptor-dependent signalling in the activation of astrocytes, we next investigated whether steroid hormone antagonists could affect the BPA-induced increase in GFAP expression in midbrain astrocyte or neuronal/glial cultures. The highly selective oestrogen receptor antagonist ICI182,780 (100 nM, 1 μM, 2 μM) was administered as pretreatment (24 h) and cotreatment (24 h) with BPA (1 pM, 1 μM) in both mouse purified midbrain astrocyte and neurone/glia cocultures. ICI182,780 failed to attenuate the activation of astrocytes induced by BPA (1 pM or 1 μM) (Fig. 5). Pretreatment (24 h) and cotreatment (24 h) with either the oestradiol receptor agonist/antagonist tamoxifen (100 nM, 1 μM or 10 μM), the progesterone receptor antagonist mifepristone (100 nM, 1 μM or 10 μM) or the androgen receptor antagonist flutamide (100 nM, 1 μM or 10 μM) failed to affect the activation of astrocytes induced by BPA (1 pM, 1 μM) in both mouse purified midbrain astrocytes (Fig. 6A–C) and neurone/glia cocultures (Fig. 6D–F). These results suggest that activation of astrocytes by BSA was not mediated via oestrogen receptors, progesterone receptors or androgen.

BPA-induced neuronal cell death

We next investigated whether *in vitro* treatment with either BPA or E_2 could induce neuronal cell death. Treatment with a high concentration of BPA (1 μM, 24 h) in mouse midbrain neurone/glia cocultures caused the robust activation of caspase-3, which is a marker of neuronal cell death (Fig. 7). Unlike BPA, a high concentration of E_2 failed to produce caspase-3 activation (Fig. 7).

Enhancement of morphine-induced rewarding effect in mice prenatally and neonatally exposed to BPA

Morphine modulates several physiological processes including a rewarding effect by stimulating opioid receptors. We previously reported that chronic treatment with morphine (3–5 mg/kg, s.c.) produced a robust place preference in mice (6, 8). However, chronic treatment with a low dose of morphine (1 mg/kg, s.c) produced neither place preference nor place aversion in control mice (Fig. 8). On the other hand, treatment with 1 mg/kg of morphine produced a significant place preference in mice whose mothers had been exposed to BPA at a dose of 200 mg/kg/day (*P < 0.05 versus control group) (Fig. 8). Treatment with morphine at 1 mg/kg also produced a significant place preference in offspring of mothers chronically treated with BPA at a dose of 3 μg/kg/day (*P < 0.05 versus control group) (Fig. 8). By contrast, treatment with morphine at 1 mg/kg failed to produce a place preference in offspring of mothers that had been chronically treated with E_2 (3 μg/kg/day) (Fig. 8).

Discussion

A growing body of evidence suggests that astrocytes are important modulators of synaptic transmission. Astrocytes can respond to neurotransmitters released within the synapse by generating elevations in intracellular Ca^{2+} concentration and release glutamate and/or ATP that signal back to neurones (18, 19). Therefore, it is worthwhile to determine the effects of BPA on astrocytes. In the present study, we investigated the dopaminergic changes in neurones and astrocytes induced by BPA.

We show here for the first time that *in vitro* treatment with BPA caused morphological changes in GFAP-positive astrocytes. In addition, this effect of BPA was biphasic: treatment with 1 pM or 1 μM of BPA caused the robust activation of astrocytes, whereas treatment with 1 nM of BPA had no detectable effect on the morphology of astrocytes.

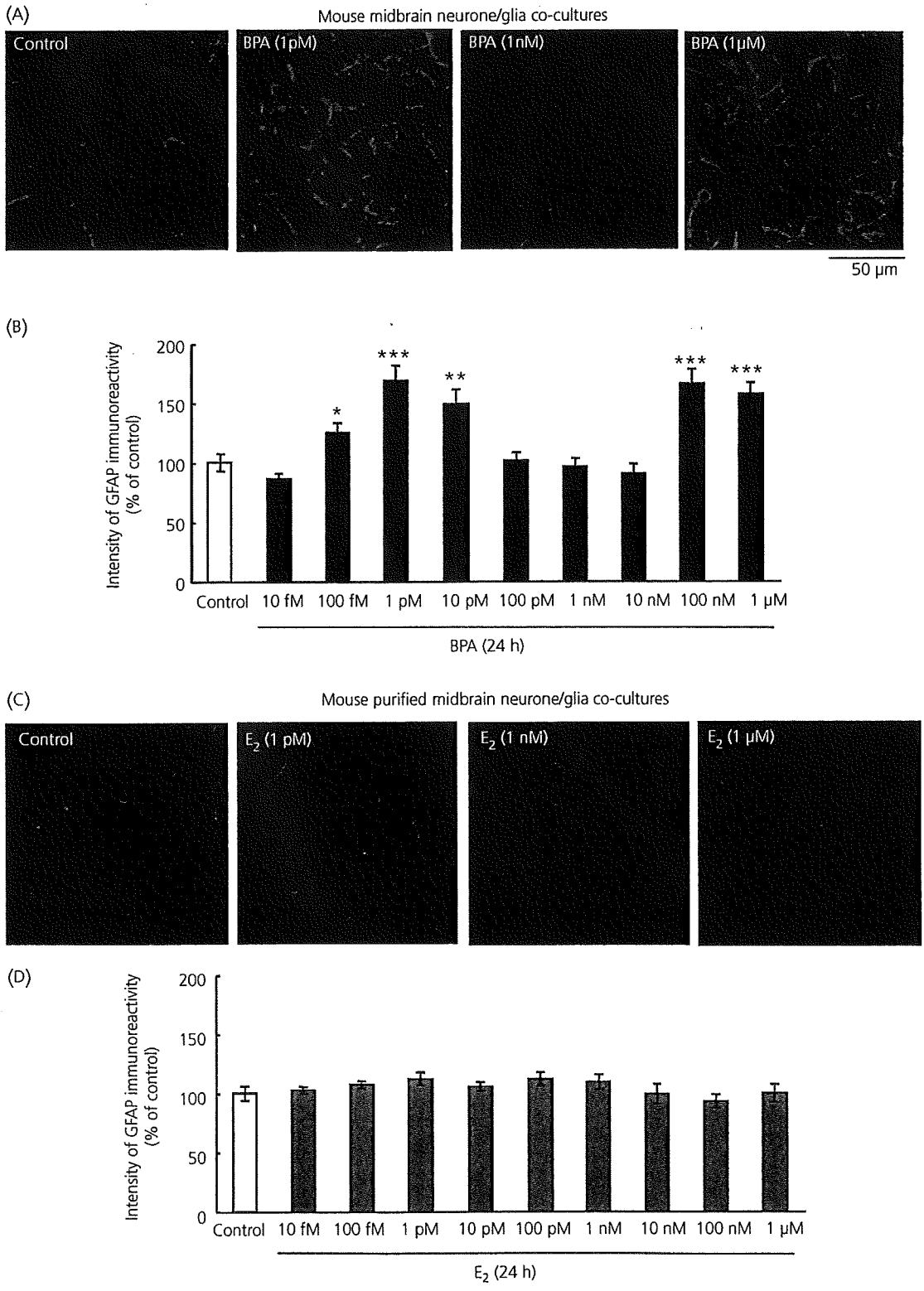
Inoue *et al.* (20) previously reported that the concentration of BPA was 0.32 ng/ml (approximately 1.4 pM) in normal human serum. Accordingly, it seems likely that 1 μM of BPA is higher than is commonly found in the environment. On the other hand, the amount of BPA that humans are exposed to results in the exposure of astrocytes to concentrations greater than 1 pM.

Neurones and astrocytes respond to various and chemical stimuli, including neurotransmitters, neuromodulators and hormones, with an increase in the intracellular Ca^{2+} concentration. These Ca^{2+} responses result from the co-ordinated activity of several molecular cascades responsible for Ca^{2+} movement into or out of the cytoplasm by way of either the extracellular space or intracellular stores. We have demonstrated that the dopamine-induced Ca^{2+} responses in mixed cultures of neurones and astrocytes were significantly enhanced by treatment with BPA (1 pM, 24 h). These findings strongly support the idea that the enhancement of Ca^{2+} responses to dopamine induced by BPA could lead to an increase in the excitability of central dopaminergic neurotransmission.

It has been reported that the stimulation of dopamine D_1 receptor increased the intracellular Ca^{2+} concentration via

FIG. 2. Treatment with bisphenol-A (BPA) for 24 h caused biphasic astrocytic activation in mouse midbrain neurone/glia cocultures. (A) Mouse midbrain neurone/glia cocultures were treated with normal medium or BPA (1 pM to 1 μM). The cells were stained with a polyclonal antibody to glial fibrillary acidic protein (GFAP). (B) Mouse midbrain neurone/glia cocultures were treated with normal medium or BPA (10 fM to 1 μM) for 24 h and stained with a polyclonal antibody to GFAP. The intensity of GFAP-immunoreactivity was measured using NIH Image. The level of GFAP-like immunoreactivity from ten areas in each image is expressed as a percent increase (mean ± SEM) with respect to that in control cells. **P < 0.001, ***P < 0.001 versus control cells. The experiments were repeatedly performed by at least three independent culture preparations. (C) Mouse midbrain neurone/glia cocultures were treated with normal medium or 17β-oestradiol (E_2, 1 pM to 1 μM). The cells were stained with a polyclonal antibody to GFAP. (D) Mouse midbrain neurone/glia cocultures were treated with normal medium or E_2 (10 fM to 1 μM) for 24 h and stained with a polyclonal antibody to GFAP. The intensity of GFAP-immunoreactivity was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells.

activation of the phospholipase C-inositol-1,4,5-triphosphate signalling pathway (21, 22). Dopamine-induced Ca^{2+} responses are also modulated by dopamine D_2 receptor (23, 24). On the other hand, dopamine D_3 receptor normally coexists with dopamine D_1 and D_2 receptors (25, 26), which contributes to the inhibitory modulation of dopamine D_1



and/or D_2 receptor-mediated signalling (7). We previously reported that prenatal and postnatal exposure to BPA (2 mg/g of mother's food) enhanced central dopamine D_1 receptor function (5) and attenuated dopamine D_3 receptor function in mice (27). Thus, the present data suggest that treatment with 1 pM of BPA may enhance dopamine D_1 receptor function and/or attenuate dopamine D_3 receptor function, resulting in enhancement of the dopamine-induced Ca^{2+} response in neurones and astrocytes.

In the present study, we observed morphological changes in astrocytes by treatment with either 1 pM or 1 μM of BPA. We also found a difference between 1 pM and 1 μM of BPA: treatment with BPA (1 pM) in midbrain neurone/glia cocultures clearly enhanced dopamine-induced Ca^{2+} responses in neurones, whereas treatment with BPA (1 μM) decreased dopamine-induced Ca^{2+} responses in neurones. Treatment with a high concentration of BPA markedly induced neuronal cell death in midbrain neurone/glia cocultures. Thus, these data suggest that a high concentration of BPA may lead to a dynamic change in the neurone-glia network, resulting in neurotoxicity.

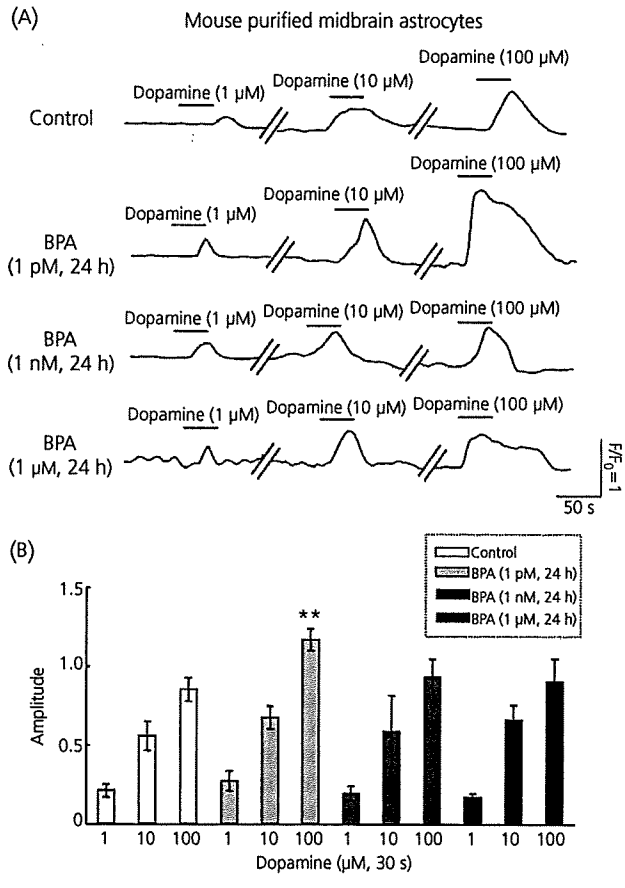


Fig. 3. The Ca^{2+} response to dopamine in astrocytes was significantly enhanced by treatment with a low concentration of bisphenol-A (BPA). (A) Traces show a typical increase in the intracellular Ca^{2+} concentration evoked by dopamine (1–100 μM) in control or BPA (1 pM, 1 nM or 1 μM) treated astrocytes. (B) The Ca^{2+} responses to dopamine (1–100 μM) in control and BPA (1 pM, 1 nM or 1 μM) treated astrocytes are summarised. Data represent the mean ± SEM of 27–63 cells. **P < 0.01 versus control cells.

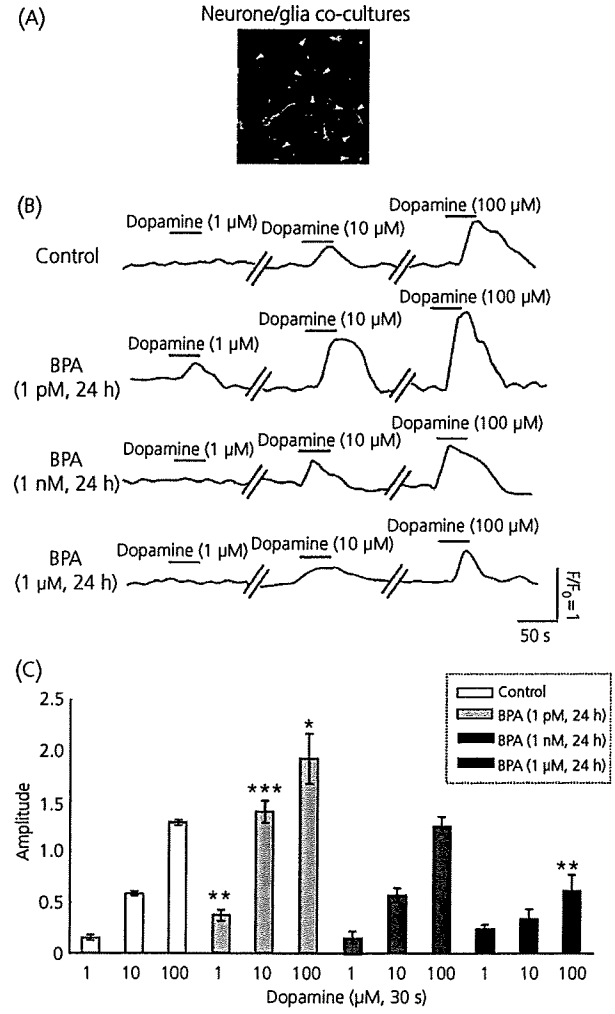


Fig. 4. The Ca^{2+} response to dopamine in neurones was significantly enhanced by treatment with a low concentration of bisphenol-A (BPA). (A) Mouse neurone/glia cocultures were stained with a mouse antibody to anti-neuronal nuclei (Neu-N) (red) and a rabbit antibody to glial fibrillary acidic protein (green). Arrow heads show Neu-N-positive neurones, which we used as cultures neurones for the Ca^{2+} imaging studies. (B) Traces show a typical increase in the intracellular Ca^{2+} concentration in control evoked by dopamine (1–100 μM) or BPA (1 pM, 1 nM or 1 μM) treated neurones. (C) The Ca^{2+} responses to dopamine (1, 10, 100 μM) in control and BPA (1 pM, 1 nM or 1 μM) treated neurones are summarised. Data represent the mean ± SEM of 27–63 cells. *P < 0.05, **P < 0.01, ***P < 0.001 versus control cells.

Similar to other drugs of abuse, the μ-opioid receptor agonist morphine acts as a rewarding stimulus when administered to animals (28). For example, rodents have been shown to intravenously self-administer morphine rather than saline when given a choice (29). Furthermore, μ-opioid receptor agonists can lower the electric current threshold for intracranial electrical self-stimulation, which indicates that opioids can facilitate the central reward mechanism itself. These positive motivational actions of opioids are indicative of their rewarding properties, and are considered to be fundamental for their ability to produce psychological dependence in humans (28).

As mentioned above, humans might be orally exposed to BPA in daily life. In a previous study, we chronically treated pregnant and lactating female mice with BPA-admixed powder food containing 2 mg of BPA/g of food, and this enhanced the development of rewarding effects induced by drugs of abuse in their adult offspring (5, 6). Under these conditions, mother mice received approximately 200 mg/kg/day of BPA. In addition, the blood level of BPA in their pups was approximately 10 ng/ml, which is considered to be more than 30-fold higher than the level for healthy human exposure (5). On the other hand, vom Saal *et al.* (30) estimated that humans are exposed to BPA at a dose of 2–20 μg/kg/day. Based on these reports, we ascertained the effects of oral exposure to BPA. A group of mother mice were orally administered BPA at a dose of 3 μg/kg/day, which is considered to be a suitable dose to reflect environmental exposure to BPA. Another group of mother mice were orally administered BPA at a dose of 200 mg/kg/day, which is considered to be higher than the environmental exposure to BPA. We also investigated the effect of prenatal and neonatal exposure to E_2 (3 μg/kg/day) to compare its effects with those of BPA. Their pups, which were prenatally and postnatelly exposed to BPA, were used in the place preference studies.

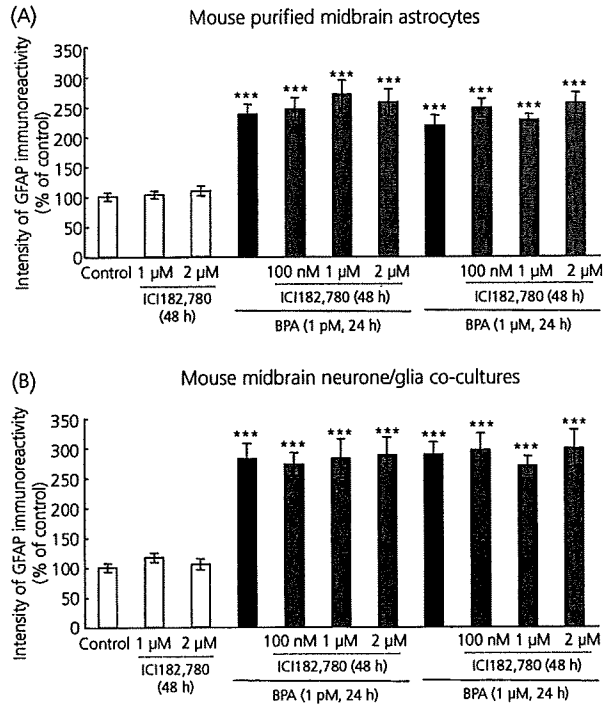


FIG. 5. The effect of ICI182,780 on astrocytic activation induced by bisphenol-A (BPA). Mouse purified midbrain astrocytes (A) or neurone/glia cocultures (B) were treated with normal medium (control) or ICI182,780 (100 nM, 1 μM or 2 μM) for 24 h. Cells were then treated with normal medium, BPA (1 pM or 1 μM) with or without ICI182,780 (100 nM, 1 μM or 2 μM) for an additional 24 h. The cells were stained with a polyclonal antibody to glial fibrillary acidic protein (GFAP). The intensity of GFAP-immunoreactivity was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells. ***$P < 0.001$ versus control cells. The white bars indicate the levels of GFAP-like immunoreactivity in the cells treated without BPA. The black bars indicate the levels of GFAP-like immunoreactivity in the cells treated with BPA. The grey bars indicate the levels of GFAP-like immunoreactivity in cells treated with BPA and ICI182,780.

One of the most important aspects of the present study was that *in vivo* prenatal and neonatal exposure to BPA (3 μg or 200 mg/kg/day administered to pregnant and lactating dams) clearly enhanced the rewarding effect of morphine in mice. Although BPA at 200 mg/kg/day may be higher than the environmental exposure, BPA may be found in the environment at a level equivalent to 3 μg/kg/day. As mentioned above, *in vitro* experiments indicate that the enhancement of Ca^{2+} responses to dopamine induced by BPA could lead to an increase in the excitability of central dopaminergic neurotransmission in both neurones and astrocytes. These findings suggest that the enhancement of dopaminergic transmission in neurones and astrocytes induced by BPA may, at least in part, lead to enhancement of the development of psychological dependence on morphine.

BPA can modulate gene transcription and numerous biological changes via oestrogenic receptors (1, 31, 32). It has been reported that equal doses of BPA and E_2 could activate the transcription factor cAMP-responsive element binding protein (CREB) via nonclassical oestrogen receptor, resulting in the transcriptional activation of CREB-responsive genes (33). On the other hand, obvious differences between BPA and E_2 have also been reported. For example, E_2 at 10 nM reduced the duration of Ca^{2+} oscillations in mouse oocytes, whereas concentrations of BPA as high as 100 μM were necessary for similar inhibition (34). It has been reported that E_2 inhibits the astrocytic uptake of glutamate, which is the most important excitatory neurotransmitter in the CNS, whereas BPA has no such effect (35). Taken together, these observations suggest that BPA and E_2 may be coupled to different signalling cascades in the CNS.

Astrocytes are among of the most important target cells for E_2. Astrocytes express all types of oestrogen receptors during development and in the adult brain (35–37). However, in the present study, neither the oestrogen receptor antagonist ICI182,780 nor the oestrogen receptor agonist/antagonist tamoxifen failed to block the activation of astrocytes induced by BPA. The progesterone receptor antagonist mifepristone and the androgen receptor antagonist flutamide also had no effect on the activation of astrocytes induced by BPA. Furthermore, E_2 had no effect on the activation of astrocytes in both purified astrocytes and neurone/glia cocultures. It appears likely that oestrogen receptors and other steroid hormone receptors may not be critical for the activation of astrocytes induced by BPA. We also found that prenatal and postnatal *in vivo* exposure to E_2 failed to enhance the rewarding effect of morphine in mice. These data suggest that oestrogenic neurotransmission is not essential for the enhancement of dopaminergic neurotransmission and hypersensitivity to the morphine-induced rewarding effect induced by exposure to BPA.

In conclusion, the results of the present study suggest that BPA induces dopaminergic amplification in neurones and astrocytes, and may contribute to potentiate the development

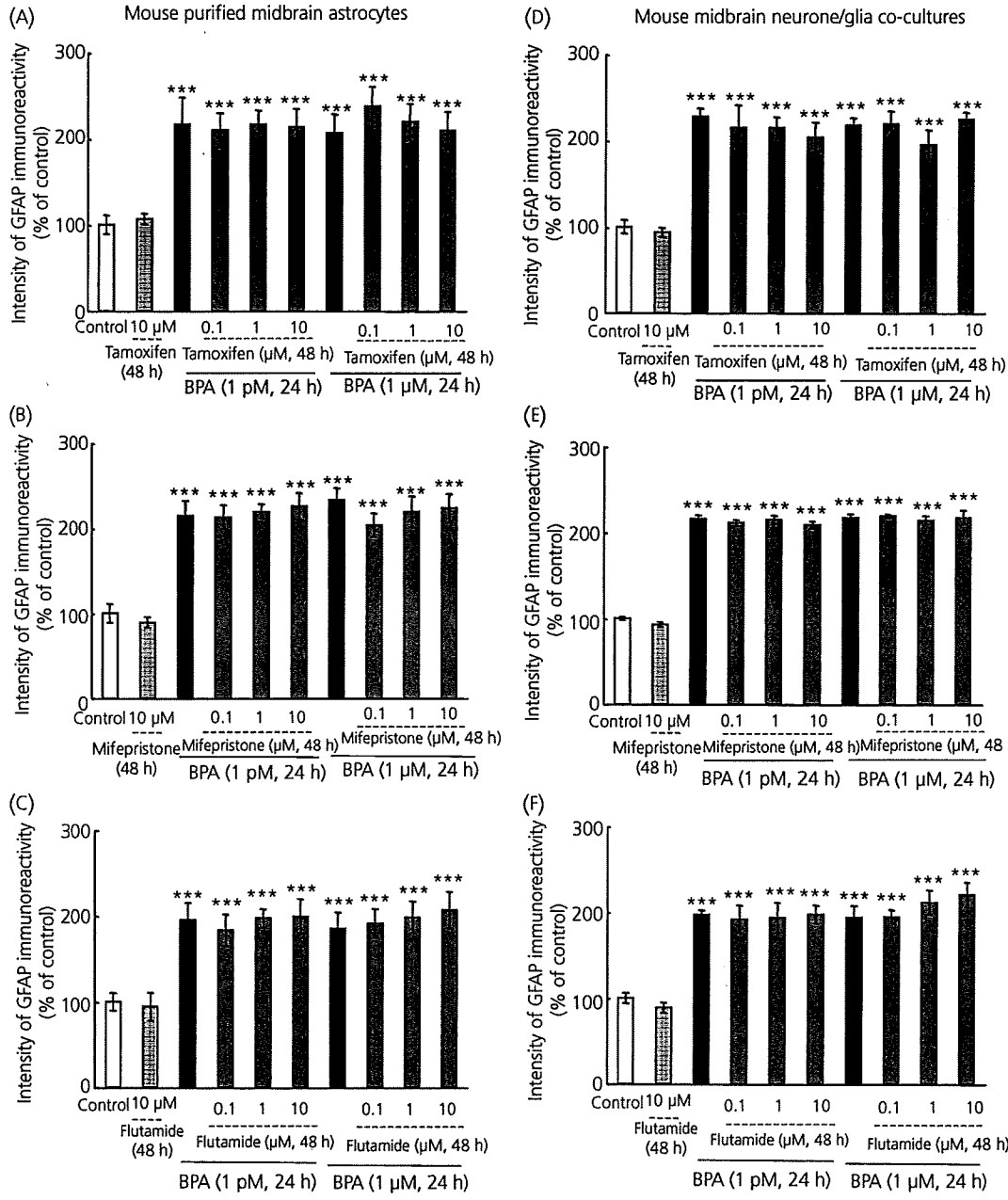


FIG. 6. Effects of steroid hormone ligands on astrocytic activation induced by bisphenol-A (BPA). Mouse purified midbrain astrocytes (A–C) or mouse midbrain neurone/glia cocultures (D–F) were treated with normal medium or tamoxifen (100 nM, 1 μM or 10 μM; A,D), mifepristone (100 nM, 1 μM or 10 μM; B,E) or flutamide (100 nM, 1 μM or 10 μM; C,F) for 24 h. Cells were then treated with normal medium, or medium that had been supplemented with BPA (1 pM, 1 μM) with or without tamoxifen (100 nM, 1 μM or 10 μM; A,D), mifepristone (100 nM, 1 μM or 10 μM; B,E) or flutamide (100 nM, 1 μM or 10 μM; C,F) for an additional 24 h. The cells were stained with a polyclonal antibody to glial fibrillary acidic protein (GFAP). The intensity of GFAP-immunoreactivity was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells. ***$P < 0.001$ versus control cells (without BPA or any antagonists). The white bars indicate the levels of GFAP-like immunoreactivity in cells treated without BPA. The black bars indicate the levels of GFAP-like immunoreactivity in cells treated with bisphenol-A. The grey bars indicate the levels of GFAP-like immunoreactivity in cells treated with BPA and steroid hormone ligands.

of the rewarding effect of morphine. Drug abuse among the young is increasing worldwide. On the other hand, emotional fragility often plays a major role in leading people to drug abuse. Our findings warn that prenatal and postnatal exposure to BPA may be linked to severe health problems in humans, including abnormalities in the CNS, resulting in an emotional sensitivity toward the development of dependence on drugs of abuse.

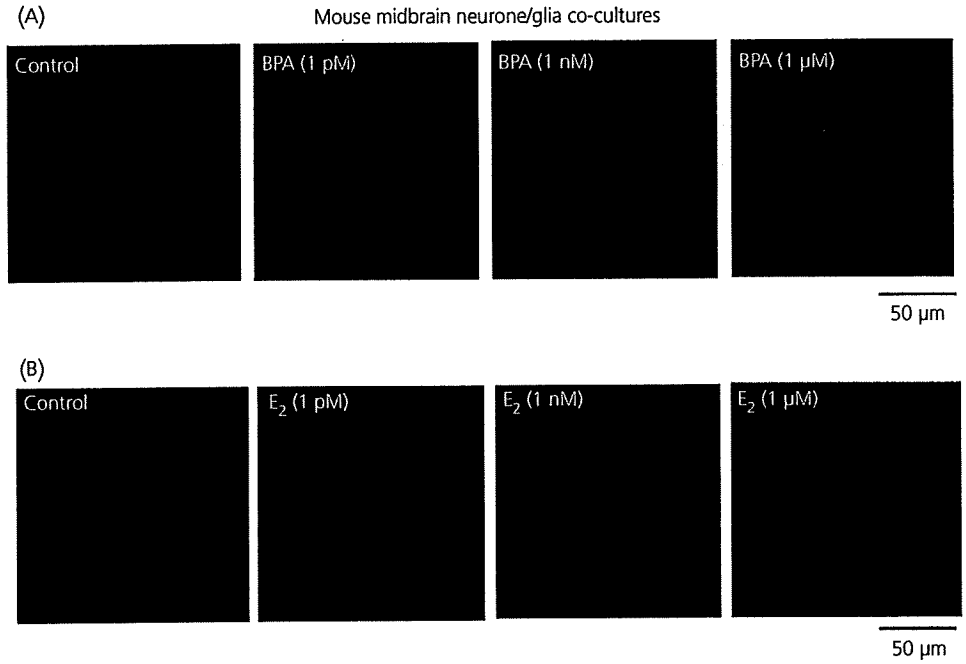


FIG. 7. A high concentration of bisphenol-A (BPA), but not 17β-oestradiol (E_2), causes a neuronal cell death in mouse midbrain neurone/glia cocultures. Mouse midbrain neurone/glia cocultures were incubated with normal medium, BPA (1 pM, 1 nM or 1 μM; A) or E_2 (1 pM, 1 nM or 1 μM; B) for 24 h. All cells were stained with a polyclonal antibody to cleaved caspase-3.

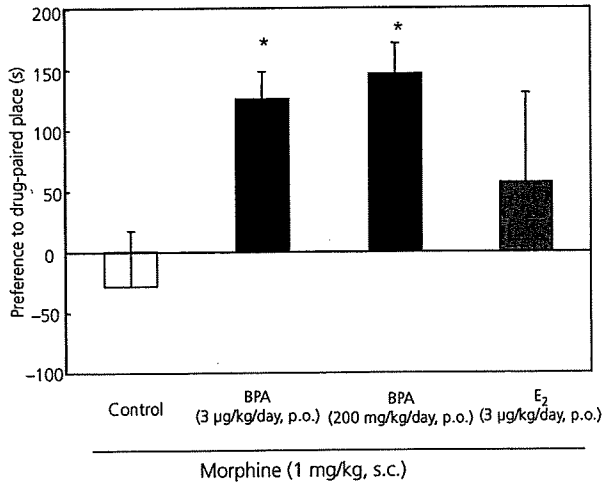


FIG. 8. Enhancement of the morphine-induced rewarding effect in mice that were prenatally and neonatally exposed to bisphenol-A (BPA). The control group did not show any place preference or place aversion with morphine (1 mg/kg s.c.). The BPA (3 μg or 200 mg/kg/day) treated group showed a significant place preference induced by morphine (*$P < 0.05$ versus control group). The 17β-oestradiol (E_2) (3 μg/kg/day) treated group did not show any place preference or place aversion with morphine. Each column represents the mean ± SEM place preference score of seven mice.

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〔特集：学会シンポジウム—環境化学物質と脳・行動〕

Bisphenol-Aの胎児期および授乳期曝露による脳内報酬系に及ぼす影響*

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（2005年4月19日受理）

要約：近年，内分泌攪乱化学物質の中枢神経系に及ぼす影響が懸念されている．本研究ではbisphenol-A（BPA）を胎児期および授乳期に曝露したマウスにおける行動影響について検討した．BPAの曝露は薬物混入飼料法に従って行った．BPAを0.002，0.5，2 mg/g of foodの濃度で処置した親から生まれたマウスをそれぞれB0.002群，B0.5群，B2群とし，BPAを曝露しないcontrol群をB0群とした．これらのマウスを用いてmorphine（MRP）あるいはmethamphetamine（METH）の報酬効果を検討した．その結果，MRPやMETHによって誘発される報酬効果はBPAの胎児期および授乳期曝露により有意に増強された．また，このようなB2群の脳内におけるD_1受容体のmRNAはB0群と比較して有意に増加していた．これらのことより，本研究で得られたBPA群におけるMRPあるいはMETH誘発報酬効果の増強にdopamine D_1受容体のup-regulationが一部関与していると考えられる．一方，これまでに当教室においてはdopamine D_3受容体の欠損が側坐核のシナプス後膜におけるdopamine受容体のシグナル伝達を増強させ，MRPの報酬効果を増強させることを報告している．そこで，BPAの胎児期および授乳期曝露によるdopamine D_3受容体の機能に及ぼす影響を検討した．その結果，BPAの胎児期および授乳期曝露により，側坐核におけるdopamine D_3受容体の機能低下が生じることを見いだした．以上の結果より，本研究で得られたBPA慢性曝露マウスにおけるMRPや覚せい剤の精神依存形成の増強は，側坐核におけるdopamine D_3受容体の機能低下ならびにdopamine D_1受容体のup-regulationに伴った中脳辺縁dopamine神経の機能亢進が一部関与している可能性が示唆される．このようなBPAの妊娠期および授乳期曝露による脳神経系への影響は，現代社会における薬物依存の増加に対する非常に重大な警鐘になりうると考えられる．

キーワード：bisphenol-A，内分泌攪乱化学物質，dopamine，報酬効果，自発運動促進作用

Bisphenol-Aは，phenolとacetoneとの縮合反応により合成され，主にポリカーボネート樹脂，エポキシ樹脂の原料としてプラスチック製の食品容器や歯科用材料などに広く使用されている．このbisphenol-Aは加熱により容易に溶出することが知られていることから，その安全性の確認が必要とされている．Bisphenol-Aの中枢神経系に対する影響については，bisphenol-Aの胎児期曝露ラットにおいて，青斑核の大きさなどの脳における性差が消失することや，雄性ラットにおける性行動の減少が報告されている（Farabollini et al, 2002; Kubo et al, 2001）．著者らもbisphenol-Aの胎児期および授乳期慢性曝露が中枢神経系に及ぼす影響を薬物依存の観点から検討し，報告してきた（Suzuki et al, 2003; Mizuo et al, 2004a, 2004b）．本稿では，bisphenol-Aの胎児期および授乳期慢性曝露マウスにおけるdopamine神経系に及ぼす影響について詳細に検討した結果を報告する．

Bisphenol-Aの胎児期および授乳期曝露がdopamine神経系に及ぼす影響

Bisphenol-Aの曝露は薬物混入飼料法に従った．Bisphenol-AをddY系雌性マウスの餌（粉末飼料）に混入し（B0：コントロール，B0.002-B2：bisphenol-A 0.002～2 mg/g of food），妊娠から離乳に至るまで母親マウスに曝露した．また，離乳後4週間以上にわたり普通飼料で通常飼育を行った後，雄性マウスを実験に使用した．まず，条件づけ場所嗜好性試験に従い，依存性薬物であるmorphine誘発報酬効果について検討した．その結果，bisphenol-Aの胎児期および授乳期慢性曝露マウスにおいて，コントロールでは報酬効果を示さない低用量のmorphine（1 mg/kg, sc）処置により，bisphenol-Aの用量依存的なmorphine誘発報酬効果の増強が認められた．次に，tilting cage法に従い，morphine（10 mg/kg, sc）誘発自発運動促進作用について検討した．その結果，bisophenol-Aの胎児期および授乳期慢性曝露マウスにおいて，morphine誘発自発運動促進作用の増強が認められた．以上のことから，bisphenol-Aの胎児期および授乳期慢性曝露により，morphineの報酬効果発現に深く関与し，腹側被蓋野から側坐核に投射している中脳辺縁dopamine神経系の機能変化が引き起こされている可能性が示唆される．Morphineは，腹側被蓋野

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* 本内容は第34回日本神経精神薬理学会，シンポジウム「環境化学物質と脳・行動」（2004年7月23日，東京）における講演の記録である．

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の dopamine 神経に投射している γ-aminobutyric acid (GABA) 神経上に存在する μ-opioid 受容体に作用することで，側坐核における dopamine の遊離を促進することが知られている (Narita et al, 2001)．そこで，著者らは次に，bisphenol-A の胎児期および授乳期慢性曝露により，腹側被蓋野における μ-opioid 受容体の機能変化が引き起こされているか否かを [^{35}S]GTPγS binding assay に従い検討した．その結果，bisphenol-A の胎児期および授乳期慢性曝露により，腹側被蓋野を含む中脳辺縁部領域において，morphine 誘発 G-protein 活性化作用に有意な変化は認められなかった．以上の結果から，bisphenol-A の胎児期および授乳期慢性曝露により，腹側被蓋野における μ-opioid 受容体の機能変化は引き起こされないことが明らかとなった．著者らはさらに，覚せい剤である methamphetamine 誘発報酬効果ならびに自発運動促進作用についても同様の検討を行った．Methamphetamine は，中脳辺縁 dopamine 神経系の神経終末に取り込まれ，直接作用し，dopamine の遊離を促進することが知られている．その結果，コントロールでは報酬効果を示さない低用量の methamphetamine (0.5 mg/kg, sc) 処置により，bisphenol-A の用量依存的な methamphetamine 誘発報酬効果の増強が認められ，さらに methamphetamine (2 mg/kg, sc) 誘発自発運動促進作用の著明な増強が認められた．また，この報酬効果の発現は，dopamine D_1 受容体拮抗薬である R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390) の前処置により完全に抑制されたことから，bisphenol-A の胎児期および授乳期慢性曝露により，側坐核における dopamine D_1 受容体の機能変化が引き起こされている可能性が示唆された．これを裏付けるために，[^{35}S]GTPγS binding assay に従い，dopamine 誘発 G-protein 活性化作用を検討した．その結果，bisphenol-A の胎児期および授乳期慢性曝露マウスより得られた側坐核を含む前脳辺縁部領域において，dopamine 誘発 G-protein 活性化作用の著明な増強が認められ，この G-protein 活性化作用は dopamine D_2 受容体拮抗薬である sulpiride の併用では抑制されず，dopamine D_1 受容体拮抗薬である SCH23390 の併用により，コントロールレベルにまで完全に抑制された．以上のことから，bisphenol-A の胎児期および授乳期慢性曝露により，側坐核における dopamine D_1 受容体の機能亢進が誘発され，その結果として依存性薬物に対する感受性の亢進が引き起こされることが初めて明らかとなった．

Bisphenol-A が dopamine 神経系に及ぼす影響の曝露時期に関する検討

脳の機能的な発達過程において，最も外界から影響を受けやすいのは胎児期から授乳期にかけてである．成体の脳は血液-脳関門が発達しており，血液から薬物など異物の脳への侵入を防いでいる．この血液-脳関門は，授乳期以降に発達することが報告されているため (Saunders and Mollgard, 1984)，胎児期から授乳期にかけては血液-脳関門がほとんど形成されておらず，未発達であると考えられる．したがって，このような時期に bisphenol-A の曝露を受けると，成体と比較して容易に bisphenol-A が脳内へ移行することが推察される．事実，すでに述べたように，著者らは bisphenol-A を胎児期から授乳期にかけて慢性的に曝露することにより，脳内 dopamine 神経系の機能に影響を及ぼすことを明らかにした．さらに，bisphenol-A は成体に対してはほとんど影響を及ぼさないことからも (Cagen et al, 1999)，胎児期から授乳期における bisphenol-A の容易な脳移行性が中枢神経系に影響を及ぼす一因となっている可能性が考えられる．

一般に，脳の発達において神経細胞の増殖は胎児期，特に器官形成期に最も盛んに行われることが明らかにされている．そのため，生後の脳重量はほとんど変化しない．一方，脳の機能的な発達，すなわちシナプスの形成およびシナプス密度の増加に伴う神経ネットワークの構築は，出生後の授乳期において最も盛んに行われていることが報告されている．著者らは，このような曝露時期の重要性を考え，bisphenol-A の曝露時期を妊娠初期，器官形成期，妊娠後期および授乳期まで 4 期に分割し，それぞれのマウスから生まれた仔を用いて，morphine 誘発自発運動促進作用および報酬効果について検討した．その結果，コントロール群と比較して，bisphenol-A の器官形成期曝露群および授乳期曝露群では morphine 誘発自発運動促進作用および報酬効果の有意な増強が認められた．さらに，前脳辺縁部領域における dopamine 誘発 G-protein 活性化作用も同様の期間において，有意な増強が認められた．これらのことから，血液-脳関門が未発達な時期であり，神経細胞の増殖および神経ネットワークの構築過程に非常に重要である器官形成期および授乳期における bisphenol-A の慢性曝露は中枢神経系に対して多大な影響を及ぼすものと考えられる．

Bisphenol-A の胎児期および授乳期曝露が dopamine D_3 受容体に及ぼす影響

Dopamine D_3 受容体は，側坐核，calleja 島および嗅結節などの辺縁系に高密度に分布することから，薬物依存との関連性が注目されている．事実，当教室において，dopamine D_3 受容体遺伝子欠損マウス (D_3KO マウス) が morphine の報酬効果を増強させることを報告している (Narita et al, 2003)．そこで，bisphenol-A の胎児期および授乳期慢性曝露群における dopamine D_3 受容体の機能変化について検討した．まず，受容体結合実験に従い，前脳辺縁部領域における dopamine D_3 受容体の変化について検討した．その結果，bisphenol-A の胎児期および授乳期慢性曝露群において，

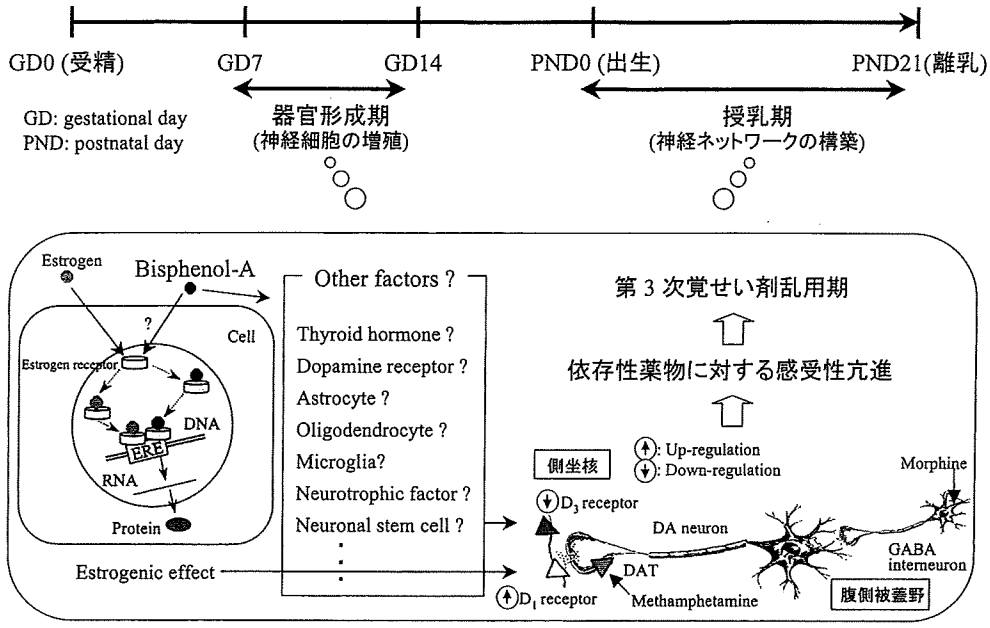

図 1　Bisphenol-A の胎児期および授乳期慢性曝露により依存性薬物に対する感受性亢進のメカニズム．Bisphenol-A の胎児期および授乳期慢性曝露により dopamine D_3 受容体の機能低下と dopamine D_1 受容体の機能亢進が引き起こされ，依存性薬物に対する感受性の亢進が引き起こされる．さらに，神経細胞の増殖のピークとなる器官形成期や神経ネットワークの構築に非常に重要な時期である授乳期における bisphenol-A 曝露が特に大きな影響を及ぼす可能性が示唆される．Bisphenol-A は estrogen 受容体（ER）に結合し，DNA 上の estrogen response element（ERE）に結合することで estrogen 様作用を有することが知られている．しかしながら，bisphenol-A の estrogen 活性は非常に弱いこと，bisphenol-A が estrogen 様作用以外の機序によりさまざまな生理作用を示すことが報告されており，著者らも estrogen 様作用以外の機序を示唆する知見を得ている．したがって，bisphenol-A の胎児期および授乳期曝露による dopamine 神経系に及ぼす影響は，bisphenol-A の有する estrogen 様作用とは異なる機序も考慮する必要がある．

dopamine D_3 受容体作動薬の放射ラベル体である［^{3}H］7-hydroxy-N,N-di-n-propyl-2-aminotetralin（［^{3}H］7-OH-DPAT）を用いて結合実験を行ったところ，Kd 値には変化が認められず，Bmax 値の有意な低下，すなわち dopamine D_3 受容体数の低下が認められた．次に，［^{35}S］GTPγS binding assay に従い，7-OH-DPAT による G-protein 活性化作用を検討した．その結果，bisphenol-A の胎児期および授乳期慢性曝露群の前脳辺縁部領域における 7-OH-DPAT 誘発 G-protein 活性化作用は，コントロール群と比較して有意な減少を示した．これらのことから，bisphenol-A の胎児期および授乳期慢性曝露により dopamine D_3 受容体の機能が特異的に減弱することが明らかとなった．一方，RT-PCR 法に従い前脳辺縁部領域および中脳辺縁部領域における dopamine D_3 受容体 mRNA の発現量を検討したところ，bisphenol-A の慢性曝露によっても有意な変化は示さなかった．これらの結果より，胎児期および授乳期の bisphenol-A 慢性曝露は dopamine D_3 受容体の新規合成に影響を与えず，dopamine D_3 受容体の代謝回転を亢進させる可能性が示唆された．

ま と め

本研究で得られた bisphenol-A の胎児期および授乳期慢性曝露マウスにおける morphine や覚せい剤の精神依存形成の増強は，dopamine D_3 受容体の機能低下に伴った dopamine D_1 受容体の機能亢進が一部関与していることを明らかにした．さらに，これらの現象は bisphenol-A 曝露を中止してから少なくとも 4 週間後に認められていることから，bisphenol-A の胎児期および授乳期慢性曝露により，不可逆的な dopamine 神経系の機能亢進が引き起こされる可能性が示唆される．近年，依存性薬物の乱用が若年層にまで広がっていること，注意欠陥多動症患者が増加していることなど，dopamine 神経系の機能異常が原因となりうる現象が大きな社会問題となっている．生命発生以来 30 億年の間に存在しなかった何万種類もの化学物質が，この 100 年間に環境中に放出されては，いくら生命体が適応能力に富んでいるとしても，それらに対応する自然界の処理能力や時間的余裕はなく，次世代のみならず，次々世代，さらに次の世代へと被害の拡大が予想される．環境化学物質がもたらす有害影響に終止符を打つのは，化学物質を環境中に放出した現代人へ課せられた義務ではないだろうか．本稿が環境化学物質問題への警鐘となり得ることを期待する．

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Abstract: Tsutomu SUZUKI, Keisuke MIZUO, Kazuya MIYAGAWA and Minoru NARITA (Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2–4–41 Ebara, Shinagawa-ku, Tokyo, 142–8501 Japan) *Exposure to bisphenol-A affects the rewarding system in mice*. Jpn. J. Neuropsychopharmacol., 25: 125–128 (2005).

Bisphenol-A has been extensively evaluated for toxicity in a variety of tests as the most common environmental endocrine disruptor. In the present study, we found that prenatal and neonatal exposure to bisphenol-A affects the development of the central dopaminergic system in the mouse limbic area. Additionally, this treatment with bisphenol-A produced a down-regulation of dopamine D_3 receptor and an up-regulation of dopamine D_1 receptor function to activate G-protein in the mouse limbic forebrain, which is thought to play a critical role for rewarding effects by drugs of abuse. We next investigated the relationship between the neurobehavioral toxicity and its exposure period. The exposure to bisphenol-A during either organogenesis or lactation, but not implantation and parturition, significantly enhanced the morphine-induced hyperlocomotion and rewarding effect. Furthermore, the exposure to bisphenol-A during either organogenesis or lactation also produced an up-regulation of dopamine D_1 receptor function to activate G-protein in the mouse limbic forebrain. These results indicate that either organogenesis or lactation is more sensitive to the bisphenol-A-induced neuronal toxicity than any other periods. In conclusion, we found here that prenatal and neonatal exposure to bisphenol-A can potentiate the central dopaminergic systems, resulting in the supersensitivity of the drugs of abuse-induced rewarding effects and hyperlocomotion in the mouse. Furthermore, the organogenesis and lactation are the most important period to cause the alteration of dopaminergic system by bisphenol-A exposure in the mouse.

Key words: Bisphenol-A, Endocrine disruptor, Dopamine, Rewarding effect, Hyperlocomotion

(Reprint requests should be sent to T. Suzuki)

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特集　物質的環境が精神機能へどう影響するか

Bisphenol-Aの胎児期および授乳期慢性曝露によるdopamine神経行動毒性発現*

● 成田　年**／宮川和也**／富田真理子**／水尾圭祐**／鈴木　勉**

Key Words : bisphenol-A, dopamine, rewarding effect, hyperlocomotion, morphine

はじめに

近年，環境中に存在するいくつかの化学物質がホルモンに類似した作用を示し，このような物質は内分泌かく乱化学物質（環境ホルモン）と呼ばれ，マスメディアを通じて広く知られることとなった．内分泌かく乱化学物質の問題が急浮上したのは，1996年にシーア・コルボーンらが著書『奪われし未来』を出版して以来のことである．しかし，この出版以前にも，生体の内分泌現象をかく乱する天然や合成化学物質の存在は知られており，世界各地で野生動物の生体異常との因果関係が疑われてきた．1970年代初頭，米国の五大湖を中心として1, 1, 1-trichloro-2, 2-bis-(monochlorophenyl)ethane(DDT)やその代謝物である1, 1-dichloro-2, 2-bis-(p-chlorophenyl)ethylene(DDE)に曝露された鳥類やワニの生殖器異常が報告された[1)~3)]．さらに，イギリスの河川では1980年代からnonylphenolが原因と考えられるローチの精巣重量減少および精子減少などの生殖異常が問題となっている[4)]．わが国においても，船底防汚塗料として用いられているtributyltinの曝露によりイボニシの雄性化がひき起こされ，その生態系に大きな影響を与えていることが報告されている[5)]．また，ヒトにおいても流産防止などの目的で使用された合成estrogenであるdiethylstilbestrol(DES)が，女性の生殖器に遅発性がんをひき起こさせることも報告されている[6)]．このような野生動物における生体異常の発見がきっかけとなって，1995年から欧米では幾多の内分泌かく乱化学物質に関する会議が開催されており，わが国においても1998年に環境庁が内分泌かく乱化学物質の対応方針を発表した．最近，内分泌かく乱化学物質は，初期に報告された"生殖器異常"といった生殖器系への影響だけではなく，中枢神経系にも影響を与える可能性が示唆されている．事実，polychlorinated biphenyl(PCB)を含んだ魚を多量に食べた母親から生まれた子供は，IQの低下や記憶力・注意力の欠陥を生じることが報告されている[7)]．一方，ヒトのみならず実験動物を用いた基礎的研究においてもPCBの曝露によって成長後に行動異常を生じることや，脳内におけるdopamineをはじめとする神経伝達物質の減少が認められることが明らかにされている[8)9)]．また，サルを用いた実験においても，PCBは次世代サルの学習行動に障害を与えることが示唆されている[10)]．

内分泌かく乱化学物質の一つであるbisphenol-Aは，phenolとacetoneとの縮合反応により合成され，主にポリカーボネート樹脂，エポキシ樹脂の原料としてプラスチック製の食品容器や歯科用材料などに広く使用されている．この

* Prenatal and neonatal exposure to bisphenol-A affects the central dopaminergic systems in mice.

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bisphenol-Aは加熱により容易に溶出することが知られていることから，その安全性の確認が必要とされている．Bisphenol-Aの中枢神経系に対する影響については，bisphenol-Aの胎児期曝露ラットにおいて，青斑核の大きさなどの脳における性差が消失することや，雄性ラットにおける性行動の減少が報告されている[11)12)]．しかしながら，bisphenol-Aの性行動以外の中枢神経系に及ぼす影響についてはほとんど検討されていないのが現状である．

本稿では，最近著者らの得た知見をもとに，内分泌かく乱化学物質であるbisphenol-Aの中枢神経系，とくにdopamine神経系に及ぼす影響について概説する．

Bisphenol-Aの胎児期および授乳期慢性曝露による依存性薬物誘発数種薬理作用に対する影響

現在，わが国では第3次薬物乱用期を迎え，依存性薬物の乱用が若年層へ浸透していることが深刻な社会問題となっている．このような依存性薬物の精神依存発現には遺伝的素因のみならず，種々の環境的素因が影響を及ぼすことが広く知られている．そこで著者らは，このような環境的素因の一つとして内分泌かく乱化学物質の影響を考え，マウスの餌にbisphenol-Aを混入し，妊娠から離乳に至るまで母親マウスに与えることで作成したbisphenol-Aの胎児期および授乳期慢性曝露マウス(B0：コントロール，B0.002-B2：bisphenol-A 0.002-2 mg/g of food)を用い，代表的な依存性薬物であるmorphineおよびmethamphetamine誘発数種薬理作用に対する影響について検討した．その結果，bisphenol-Aの胎児期および授乳期慢性曝露群においてはコントロール群と比較してmorphineおよびmethamphetamine誘発自発運動促進作用および報酬効果(精神依存)の有意な増強(図1)，さらには，methamphetamine間欠投与誘発逆耐性形成の増強がひき起こされることが初めて明らかになった(図2)．

この依存性薬物の精神依存形成や発現には腹側被蓋野から側坐核に投射している中脳辺縁dopamine神経系が深く関与していることが広く知られている．そこで，bisphenol-Aのdopamine神経系に及ぼす影響について詳細に検討した．まず，dopamine受容体の機能変化を検討する目的で，[^{35}S]GTPγS binding assayを用い，側坐核を含む領域である前脳辺縁部におけるdopamine誘発Gタンパク質活性化作用について検討を行ったところ，bisphenol-Aの胎児期および授乳期慢性曝露群においてはコントロール群と比較して有意なdopamine誘発Gタンパク質活性化作用が認められた(図3-A)．また著者らは，このbisphenol-Aの胎児期および授乳期慢性曝露によるdopamine誘発Gタンパク質活性化作用の増強とdopamine受容体サブタイプとの関連を検討する目的で，選択的dopamine D_1受容体拮抗薬であるR(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2, 3, 4, 5-tetrahydro-1H-3-benzazepine(SCH23390)ならびに選択的dopamine D_2受容体拮抗薬sulpirideを用いて拮抗試験を行った(図3-B)．その結果，bisphenol-Aの胎児期および授乳期慢性曝露群で認められたdopamine誘発Gタンパク質活性化作用の増強は，SCH23390の併用によりコントロール群と同程度まで抑制された．しかしながら，sulpirideの併用ではbisphenol-Aの慢性曝露によってひき起こされるGタンパク質活性化作用の増強が維持されていた．これらのことから，bisphenol-A慢性曝露によるdopamine受容体の機能亢進は，主にdopamine D_1受容体の機能亢進に由来する可能性が考えられる．そこで，RT-PCR法に従い，全脳におけるdopamine D_1受容体mRNAの発現量を検討したところ，bisphenol-Aの胎児期および授乳期慢性曝露群において，コントロール群と比較してdopamine D_1受容体mRNA量の有意な増加が認められた．

一方，dopamine D_3受容体は，側坐核，calleja島および嗅結節などの辺縁系に高密度に分布することから，薬物依存との関連性が注目されている．事実，当教室において，dopamine D_3受容体遺伝子欠損マウス(D_3KOマウス)がmorphineの報酬効果を増強させることを報告している[13)]．そこで，bisphenol-Aの胎児期および授乳期慢性曝露群におけるdopamine D_3受容体の機能変化について検討した．まず，受容体結合実験に従い，前脳辺縁部領域におけるdopamine D_3受容体の変化について検討した．その結果，bisphenol-Aの

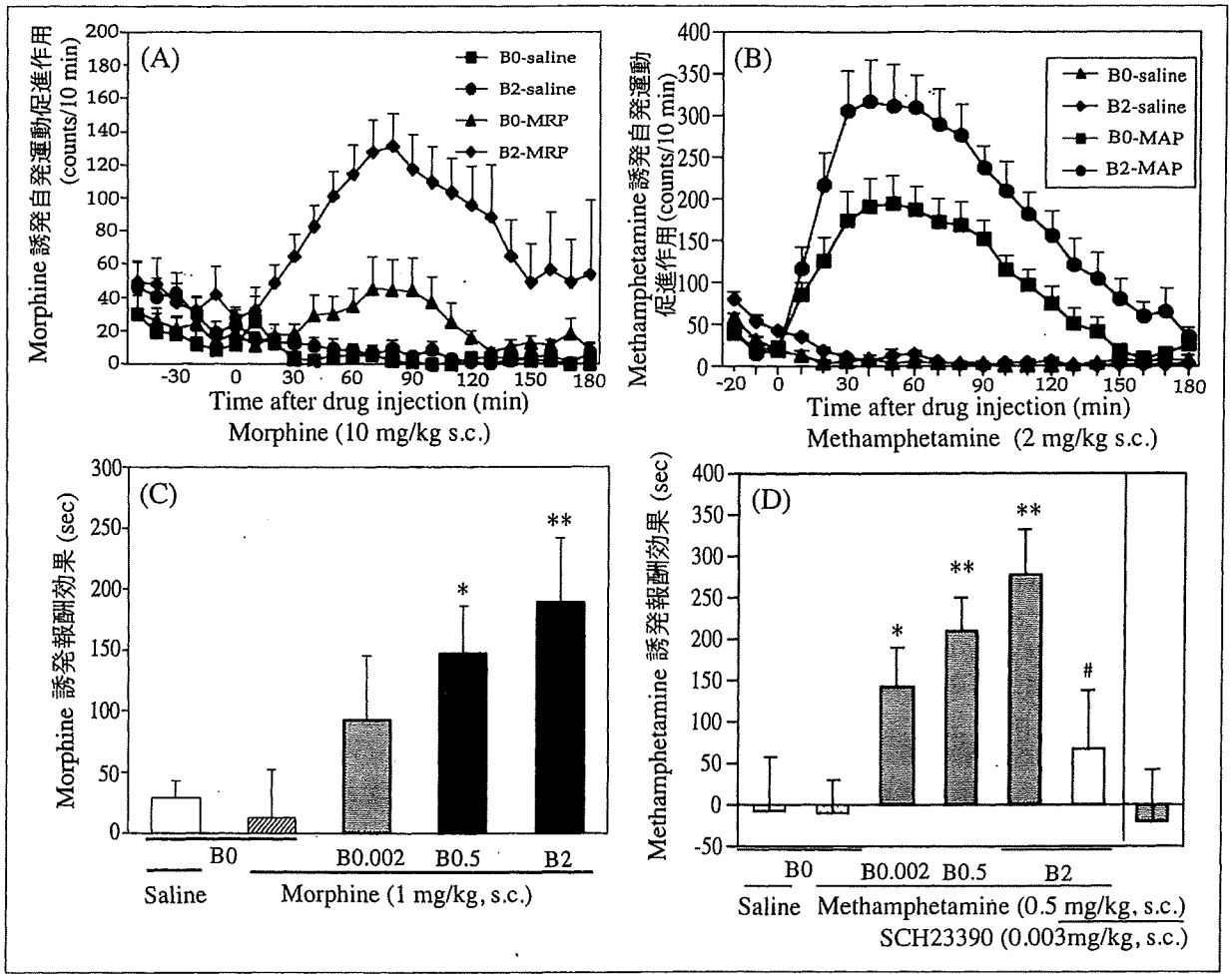


図1 Bisphenol-Aの胎児期および授乳期慢性曝露によるmorphine(A)およびmethamphetamine(B)誘発自発運動促進作用とmorphine(C)およびmethamphetamine(D)誘発報酬効果(精神依存)に対する影響

Bisphenol-Aの胎児期および授乳期慢性曝露により，morphine(10mg/kg, s.c.)誘発自発運動促進作用(A)およびmethamphetamine(2 mg/kg, s.c.)誘発自発運動促進作用(B)の有意な増強が認められた．Bisphenol-Aの胎児期および授乳期慢性曝露群により，コントロール群で報酬効果が認められない用量のmorphine(C；1 mg/kg, s.c.)およびmethamphetamine(D；0.5mg/kg, s.c.)において，bisphenol-Aの用量依存的な報酬効果増強が認められた．このmethamphetamine誘発報酬効果は，dopamine D_1受容体拮抗薬であるSCH23390(0.003mg/kg, s.c.)の前処置により抑制された．(A)Each point represents the mean activity counts for 10min with S.E.M. of 9～10mice. $F_{1,340}=6.617$, $p<0.05$ vs. B0 group : triangle. (B)Each point represents the mean activity counts for 10 min with S.E.M. of 9～10mice. $F_{1,340}=6.617$, $p<0.05$ vs. B0 group ; square. (C)Each column represents the mean place preference score with S.E.M. of 6～10mice. $*p<0.05$, $**p<0.01$ vs. B0 group. (D)Each column represents the mean place preference score with S.E.M. of 4～10mice. $*p<0.05$, $**p<0.01$ vs. B0 group, $\#p<0.05$vs. B2-methamphetamine group.

胎児期および授乳期慢性曝露群において，Bmax値の有意な低下，すなわちdopamine D_3受容体数の低下が認められた(図4-A～C)．次に，[^{35}S]-GTPγS binding assayに従いdopamine D_3受容体作動薬である7-hydroxy-N, N-di-n-propyl-2-aminotetralin(7-OH-DPAT)によるGタンパク質活性化作用を検討した．その結果，bisphenol-Aの胎児期および授乳期慢性曝露群の前脳辺縁部領域における7-OH-DPAT誘発Gタンパク質活性化作用は，コントロール群と比較して有意な減少を示した(図4-D)．これらの条件下，前脳辺縁部領域におけるdopamine D_2受容体作動薬N-propylnorapomorphine(NPA)誘発Gタンパク質活性化作用は，bisphenol-Aの胎児期および授乳期慢性曝露により影響を受けなかった．これらのことから，bisphenol-Aの胎児期および授乳期慢性曝露によりdopamine D_3受容体の機能が特異的に減弱することが明らかとなった．一方，RT-

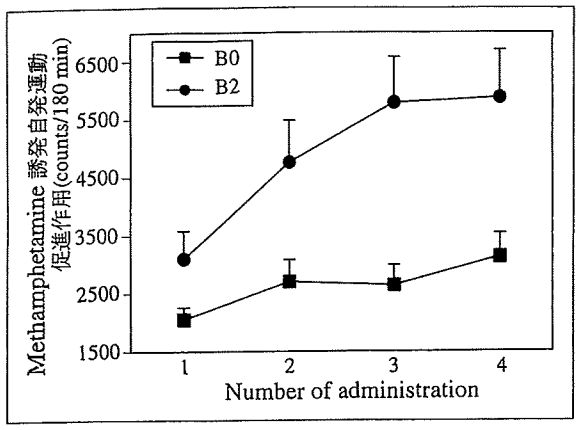


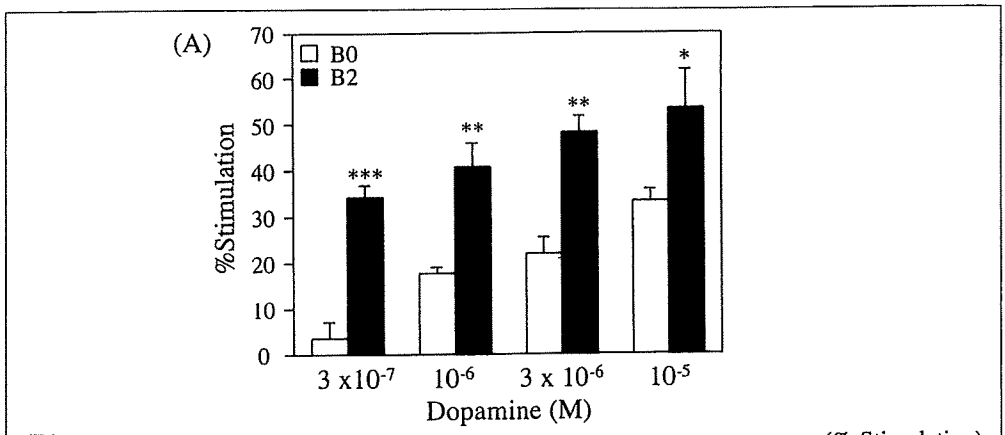
図2 Methamphetamine間欠投与による自発運動促進作用に対する逆耐性形成に及ぼすbisphenol-A胎児期および授乳期慢性曝露の影響

Bisphenol-A胎児期および授乳期慢性曝露により，methamphetamine間欠投与による逆耐性形成の有意な増強が認められた．Each point represents the mean total activity counts for 180min with S.E.M. of 9～10mice. $F_{1,54}=9.459$, $p<0.01$ vs B0 group.

PCR法に従い前脳辺縁部領域および中脳辺縁部領域におけるdopamine D_3受容体mRNAの発現量を検討したところ，bisphenol-Aの慢性曝露によっても有意な変化は示さなかった．これらの結果より，胎児期および授乳期のbisphenol-A慢性曝露は受容体の新規合成に影響を与えず，dopamine D_3受容体の代謝回転を亢進させる可能性が示唆された．このように著者らの研究により，内分泌かく乱化学物質であるbisphenol-Aの胎児期および授乳期慢性曝露が，中枢神経系，とくにdopamine神経系に直接的な影響を及ぼすという非常に興味深い事実が明らかとなった．

Bisphenol-Aの作用機序

Bisphenol-Aはestrogen様作用を有することが示唆されている[14)~16)]．近年，dopamine D_1受容体の遺伝子上にはestrogen responsive element (ERE)が存在することが明らかとなり，estrogen処置によりdopamine D_1受容体mRNAのup-regulationがひき起こされることが報告されている[17)]．このことから，bisphenol-Aの胎児期および授乳



(B) (% Stimulation)

	Dopamine (10^{-5}M)	Dopamine (10^{-5}M) +SCH23390 (10^{-5}M)	Dopamine (10^{-5}M) +Sulpiride (10^{-5}M)	SCH23390 (10^{-5}M)	Sulpiride (10^{-5}M)
B0	28.8±1.9	20.7±3.5	5.6±1.5 ###	5.3 ±1.0	-8.2±1.8
B2	48. 9±2.3***	22.3±2.9 ###	18.9±1.7***, ###	7.6 ±3.1	-1.2±3.0

図3 Bisphenol-Aの胎児期および授乳期慢性曝露による(A)dopamine誘発Gタンパク質活性化作用に対する影響および(B)その効果におけるdopamine受容体サブタイプの関与

(A)Bisphenol-Aの胎児期および授乳期慢性曝露により，前脳辺縁部領域におけるdopamine誘発Gタンパク質活性化作用の有意な増強が認められた．(B)Bisphenol-Aの胎児期および授乳期慢性曝露によりひき起こされた前脳辺縁部におけるdopamine誘発Gタンパク質活性化作用の増強はdopamine D_1受容体拮抗薬であるSCH23390の併用により抑制されたが，dopamine D_2受容体拮抗薬であるsulprideの併用によっては抑制されなかった．(A)Each column represents the mean with S.E.M. of 3 independent samples. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. B0 group. (B)Each value represents the mean with S.E.M. of 3 independent samples. ***$p<0.001$ vs. B0 group, ###$p<0.001$ vs. dopamine alone.